Antibodies to liposomal phosphatidylcholine and phosphatidylsulfocholine[1]

NABILA M. WASSEF,[2] GLENN M. SWARTZ, JR., AND CARL R. ALVING
Department of Membrane Biochemistry, Walter Reed Army Institute of Research, Washington, D.C. 20307-5100, U.S.A.
AND
MORRIS KATES
Department of Biochemistry, University of Ottawa, Ottawa, Ont., Canada K1N 9B4

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Antibodies against dimyristoyl phosphatidylsulfocholine or dimyristoyl phosphatidylcholine were raised in rabbits after injection of liposomes containing phosphatidylsulfocholine or phosphatidylcholine, cholesterol, and lipid A. The antibody activities were assayed by complement-dependent immune damage to liposomes and by a solid-phase, enzyme-linked immunosorbent assay using purified dimyristoyl phosphatidylcholine or dimyristoyl phosphatidylsulfocholine as antigen. Each antiserum raised against phosphatidylsulfocholine reacted with liposomes containing phosphatidylcholine, and each antiserum raised against phosphatidylcholine reacted with liposomes containing phosphatidylsulfocholine. However, adsorption of dimyristoyl phosphatidylsulfocholine antiserum with liposomes containing dimyristoyl phosphatidylcholine removed all activity against dimyristoyl phosphatidylcholine, but did not eliminate antibody activity against dimyristoyl phosphatidylsulfocholine. These results indicate that the antiserum against phosphatidylsulfocholine contained mixed populations of antibodies. Polyclonal antisera that have been appropriately adsorbed can therefore be obtained with a high degree of specificity for phosphatidylsulfocholine and such antisera can distinguish between phosphatidylsulfocholine and phosphatidylcholine.

Key words: liposomes, antibodies, phosphatidylsulfocholine, phosphatidylcholine.

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L'injection à des lapins de liposomes composés de la phosphatidylsulfocholine ou de la phosphatidylcholine, de cholestérol et de lipide A produit des anticorps dirigés respectivement contre la dimyristoyl phosphatidylsulfocholine ou la dimyristoyl phosphatidylcholine. Nous avons évalué le titre de ces anticorps par la mesure des lésions causées aux liposomes par les anticorps et le complément et par un test immunoenzymatique ELISA utilisant les phospholipides purifiés comme antigènes. Chaque anticorps développé contre la phosphatidylsulfocholine a réagi avec les liposomes renfermant la phosphatidylcholine et chaque anticorps développé contre la phosphatidylcholine a réagi avec les liposomes contenant la phosphatidylsulfocholine. Toutefois, l'adsorption d'un des antisérum dirigé contre la dimyristoyl phosphatidylsulfocholine avec des liposomes contenant la dimyristoyl phosphatidylcholine a permis d'éliminer l'activité de cet antisérum avec la dimyristoyl phosphatidylcholine mais non son activité avec la dimyristoyl phosphatidylsulfocholine. Ces résultats montrent que l'antisérum contre la phosphatidylsulfocholine renferme non pas une seule population, mais un mélange de plusieurs populations d'anticorps. Il est donc possible qu'un antisérum polyclonal convenablement adsorbé puisse résulter en un antisérum doué d'un fort degré de spécificité envers la phosphatidylsulfocholine et capable de distinguer la phosphatidylsulfocholine de la phosphatidylcholine.

Mots clés : liposomes, anticorps, phosphatidylsulfocholine, phosphatidylcholine.

[Traduit par la revue]

Introduction

Most phospholipids are immunogenic and allow formation of antibodies directed against their polar head groups (Inoue and Nojima 1967; Kataoka and Nojima 1970; Guarnieri and Eisner 1974). In the studies cited above the antibodies were induced by immunizing with emulsions containing protein–phospholipid conjugates in the presence of an adjuvant such as complete Freund's adjuvant (reviewed by Alving 1977). In recent years we have demonstrated that polyclonal or monoclonal antibodies against liposomal phospholipids can be raised by injecting liposomes containing LA as adjuvant into rabbits or mice (Schuster *et al.* 1979; Alving *et al.* 1980; Banerji and Alving 1981; Banerji *et al.* 1982; Wassef *et al.* 1984; Alving 1986).

In the present paper, we report on the production of polyclonal "antiliposome" antisera having reactivities with DMPC and DMPSC. PSC is a sulfonium analog of PC, which is found in certain marine diatoms and algae (Bisseret *et al.* 1984). This sulfolipid was identified and synthesized by Kates and his colleagues and its physical properties were extensively investigated (Tremblay and Kates 1979, 1981; Bittman *et al.* 1984). Figure 1 shows the chemical structures of DMPC and DMPSC that were the phospholipid antigens used in the immunizing liposomes.

Because of the close resemblance of DMPSC to DMPC, the question arose whether all of the antibodies in polyclonal

ABBREVIATIONS: LA, lipid A; DMPC, dimyristol phosphatidylcholine; DMPSC, dimyristoyl phosphatidylsulfocholine; PSC, sulfonium analog of phosphatidylcholine; PC, phosphatidylcholine; CHOL, cholesterol; DCP, dicetyl phosphate; TLC, thin-layer chromatography; GLC, gas–liquid chromatography; GLU CER, glucosyl ceramide; ELISA, enzyme-linked immunosorbent assay; GPS, guinea pig serum; PBS, phosphate-buffered saline; FBS, fetal bovine serum; IgG (H + L), immunoglobulin G (heavy + light chains); PIP, phosphatidylinositol phosphate; DPPC, dipalmitoyl phosphatidylcholine.

[1]Nabila Wassef, Glenn Swartz, and Carl Alving would like to dedicate this paper to their coauthor Dr. Morris Kates in recognition of his valuable contributions to the field of lipid biochemistry.

[2]Author to whom all correspondence should be addressed.

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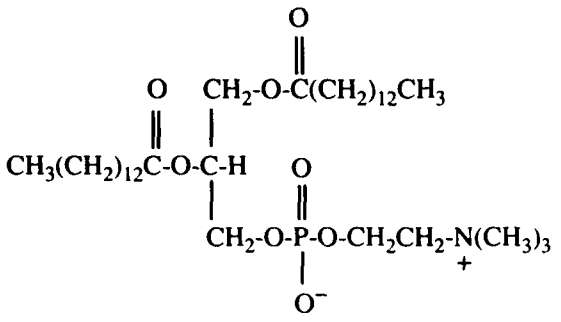
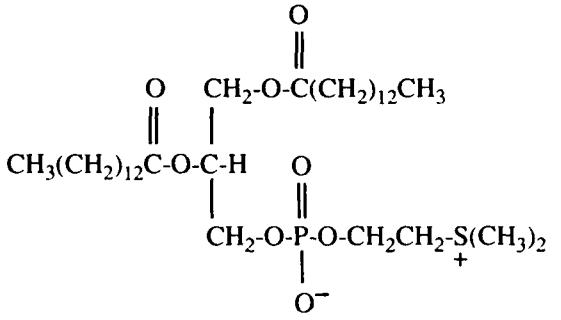


Dimyristoylphosphatidylcholine



Dimyristoylphosphatidylsulfocholine

FIG. 1. Chemical structures of DMPC and DMPSC.

antisera cross-react with both antigens. The goal was to determine if polyclonal antisera could be obtained that would differentiate the subtle differences between these phospholipids. Such an antiserum might be useful as a tool for detecting DMPSC in crude samples or during purification procedures.

Materials and methods

Source of lipids

Lipids were purchased from the following sources: DMPC, Sigma Chemical Co. St. Louis, MO; CHOL, Calbiochem, La Jolla, CA; DCP, K and K Laboratories, Plainview, NY; LA (containing 1.1 μg/nmol LA phosphate) from *Salmonella minnesota* R595, List Biological Labs., Campbell, CA. DMPSC was synthesized as described elsewhere (Tremblay and Kates 1979) and finally purified by chromatography on a column of silicic acid eluted with chloroform–methanol (1:1, v/v), followed by acetone precipitation. DMPSC showed a single spot on TLC in chloroform – methanol – concentrated ammonium hydroxide (65:35:5, by volume). Fatty acid analysis by GLC showed the presence only of myristic acid (99.8%).

Preparation of liposomes

Complete details of liposome preparation are reviewed elsewhere (Alving *et al.* 1984). Briefly, multilamellar liposomes were prepared from a mixture of DMPSC or DMPC, CHOL, and DCP in molar ratios of 2:1.5:0.22. Liposomes used for immunization lacked DCP, but contained 20 nmol of LA phosphate (~22 μg LA)/μmol phospholipid (Schuster *et al.* 1979). Liposomes used for the complement-dependent immune damage assay contained DCP and also 12.5 nmol LA phosphate (~14 μg LA)/μmol phospholipid where indicated. When GLU CER was incorporated in the liposomes, it was at a concentration of 150 μg/μmol phospholipid.

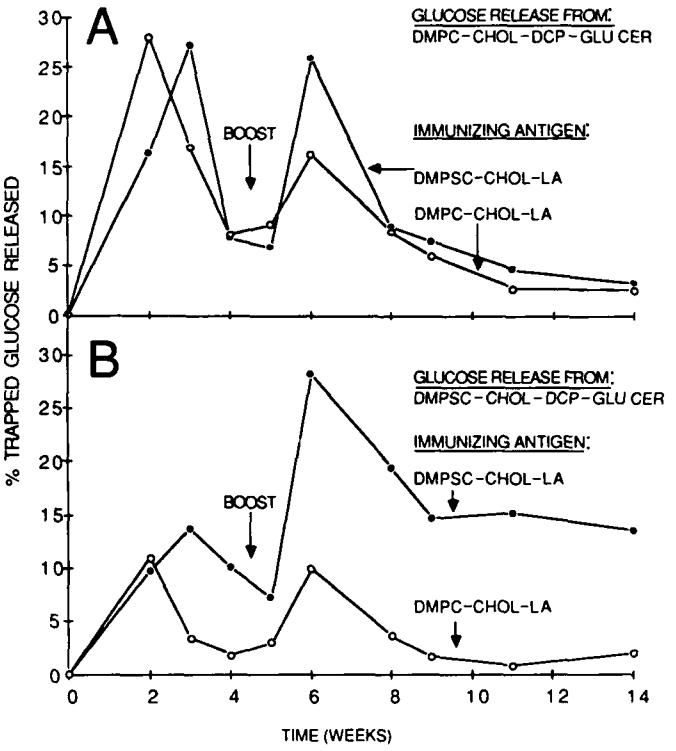


FIG. 2. Time course of antibody activity after injection of liposomes composed of DMPC-CHOL-LA (○) or DMPSC-CHOL-LA (●). Each line represents the average of two rabbits immunized and bled at the indicated times. Complement-mediated immune damage against liposomes containing DMPC is shown in A and against liposomes containing DMPSC is shown in B. The data shown have been corrected for background activity by subtracting the values obtained for preimmune sera.

The lipids in chloroform were dried together in a pear-shaped flask on a rotary evaporator, followed by 1 h under high vacuum in a desiccator. The dried lipids were then dispersed in the appropriate swelling solution by vigorous vortexing so that the final concentration of liposomal DMPC or DMPSC was always 10 mM. The swelling solution for liposomes used for immunization was 0.154 M NaCl, and the swelling solution for liposomes used in the complement-dependent immune damage assay was 0.308 M glucose.

Immunization schemes

Male New Zealand white rabbits (2.5–3.0 kg) were immunized with 1.0 mL of liposomes containing 10 μmol of phospholipids. Each rabbit received a 0.5-mL dose, injected s.c. or i.m. at the caudal muscle of each thigh. The rabbits were immunized, bled, and boosted at the time intervals indicated in the respective figures. The following liposome compositions were used for immunization: group 1, DMPSC-CHOL-LA; group 2, DMPC-CHOL-LA. Each group was comprised of two rabbits.

Assays of antibody activities

Antibody activities against DMPSC or DMPC were assayed both by complement-dependent immune damage to liposomes and by ELISA.

Complement-dependent immune damage to liposomes measured the release of trapped glucose from liposomes containing DMPSC or DMPC owing to antibody-mediated complement-dependent damage to test liposomes. The assay has been described in detail elsewhere (Alving *et al.* 1984). Briefly, release of trapped liposomal glucose was enzymatically measured in a total volume of 1 mL, using a Tris-buffered assay solution containing hexokinase, glucose-6-phosphate dehydrogenase, ATP, NADP, and Ca^{2+} and

Mg^{2+}. Each 5-μL aliquot of liposomes was incubated at room temperature with either 30 μL of rabbit antiserum from different bleedings (Fig. 2) or with increasing volumes of rabbit antiserum from the same bleeding (Fig. 4), and with 100 μL of GPS as a source of complement. Glucose release was detected after 30 min at room temperature by increased A_{340} owing to reduction of the NADP. Data were expressed as percentage of trapped glucose released and a value of 5% glucose released represented a threshold for positive activity. The total amount of glucose originally trapped in the liposomes was determined by disrupting the liposomes with chloroform.

The general method for performing an ELISA with a lipid antigen has been described by Swartz *et al.* (1988). Appropriate lipid antigens were used to coat the wells of polystyrene plates (Immulon II, "U" bottom, Dynatech Laboratories, Alexandria, VA). Lipids were used at a concentration of 1 nmol per well with the exception of CHOL, which was used at a concentration of 1 μg per well. The ethanolic solution of lipid was evaporated, and the plates were further dried under high vacuum for 10 min. Plates were blocked with 110 μL per well of PBS containing 10% heat-inactivated FBS (Gibco Laboratories, Grand Island, NY). Plates were blocked for 1 h at room temperature or overnight at 4°C. Fifty microlitres of rabbit antiserum diluted in PBS containing 10% FBS was added to the wells and incubated 3 h at room temperature or overnight at 4°C. Plates were then washed three times, for 5 min each, with PBS alone. Goat anti-rabbit IgG (H + L) - alkaline phosphatase conjugate (Kirkegaard and Perry Laboratories, Gaithersburg, MD) diluted in PBS containing 10% FBS was added at a concentration of 25 ng per well. Following 1 h of incubation at room temperature, the plates were again washed three times, for 5 min each, with PBS. Fifty microlitres of *p*-nitrophenyl phosphate (Kirkegaard and Perry Laboratories, Gaithersburg, MD) in diethanolamine buffer (2 mg/mL) was added to the wells and incubated for 1 h at room temperature in the dark. Absorbance at 405 nm was measured using a UVmax microplate reader (Molecular Devices, Palo Alto, CA). Values reported were adjusted by subtracting values in wells that contained antiserum but lacked antigen.

Results

Antibodies to DMPC and DMPSC

The production of anti-DMPC or anti-DMPSC antiserum, as determined by complement damage to liposomes containing DMPC or DMPSC, is shown in Figs. 2A and 2B, respectively. Anti-DMPSC serum apparently cross-reacted with liposomes containing DMPC (Fig. 2A), and anti-DMPC serum apparently cross-reacted with liposomes containing DMPSC (Fig. 2B). Anti-DMPC serum activity and cross-reactivity against DMPC in anti-DMPSC serum reached peaks at 2–3 weeks (Fig. 2A). When the sera were tested with liposomes containing DMPSC, the activity of anti-DMPSC serum against DMPSC was much higher and more prolonged after boosting than the cross-reacting activity in anti-DMPC serum (Fig. 2B). Despite the differences observed, the data indicated that the patterns of antibody activities were similar for antibodies elicited by DMPC and DMPSC, and there appeared to be extensive cross-reactivity between both groups. However, from the data presented it was impossible to determine if reactivity of an antiserum with both antigens was due to cross-reactivity or to mixtures of monospecific antibodies.

It should be pointed out that GLU CER was included in the test liposomes because of previous work with "anti-liposome" antibodies that showed that the presence of ceramide in the test liposomes nonspecifically increased the amount of glucose released from the liposomes and

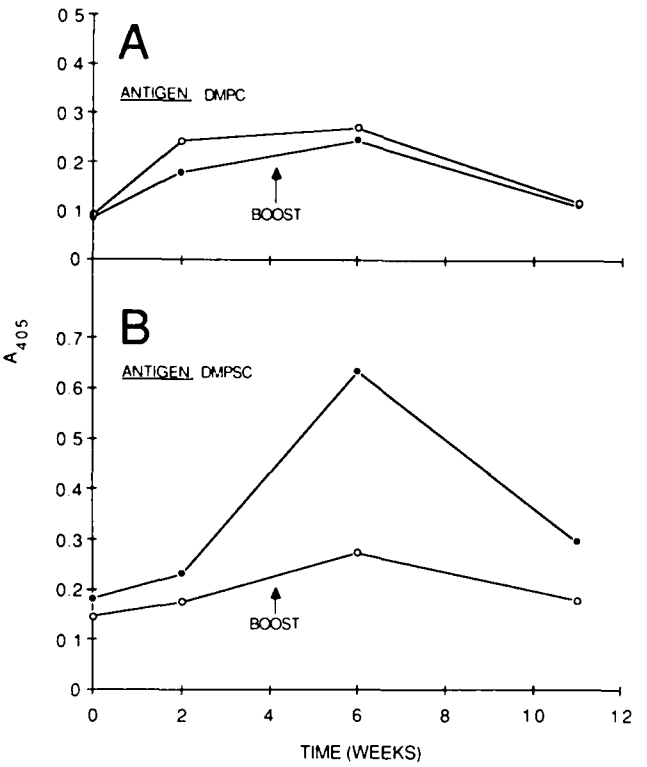


FIG. 3. ELISA determinations of the activities of rabbit antisera against either DMPC of DMPSC. Each panel shows the activities of sera obtained from rabbits immunized with liposomes composed of DMPC-CHOL-LA (○) or DMPSC-CHOL-LA (●) at the times indicated. Values reported are the average of two rabbits. Sera were tested at a dilution of 1:25.

increased the sensitivity of the glucose release method for detecting antibodies (Banerji and Alving 1981). This is a nonspecific amplification technique that does not indicate the presence of antibodies to GLU CER.

Immune reactivities with purified antigens

A solid-phase ELISA employing either purified DMPC or DMPSC as the antigen demonstrated that antibodies that bound to either of these two phospholipids could be obtained (Fig. 3). Animals immunized with liposomes containing DMPC had similar reactivities to both DMPSC and DMPC (Fig. 3A). In contrast, animals immunized with liposomes containing DMPSC showed higher binding to DMPSC compared with DMPC (Fig. 3B). All animals had detectable binding to both DMPSC and DMPC when comparisons were made with preimmunization serum samples which were barely detectable.

Adsorption studies

The specificity of one of the antisera to DMPSC was further examined by adsorption of the antiserum with liposomes containing DMPC. The antiserum that was obtained at 6 weeks after immunization was adsorbed with liposomes containing DMPC-CHOL-DCP-LA. This antiserum originally had shown substantial reactivity with liposomes containing DMPC at 6 weeks (Figs. 2 and 3), but following adsorption the antibody activity against DMPC was completely removed while the antibody activity against DMPSC was not affected, when tested by complement-dependent immune damage to liposomes (Fig. 4). When the antiserum was tested by ELISA using individual liposomal

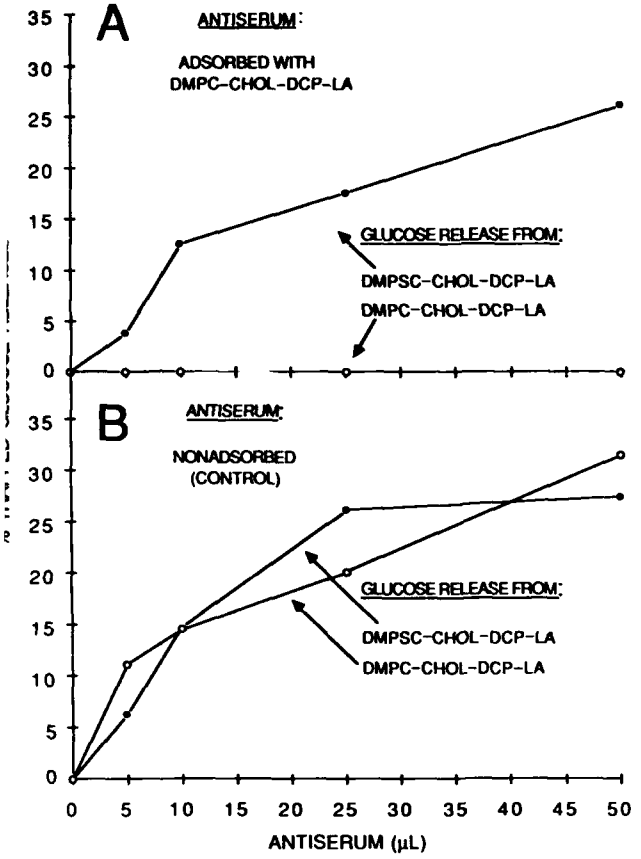


FIG. 4. Adsorption of antibody activity against liposomes containing DMPC. Antiserum (1 mL) from a rabbit immunized with liposomes containing DMPSC (6 weeks bleeding, see Figs. 2 and 3) was incubated for 30 min at room temperature with 0.4 mL of liposomes composed of DMPC-CHOL-DCP-LA. The liposomes were removed by centrifugation (27 000 × *g*, 10 min). Glucose release from liposomes containing DMPC (○) or DMPSC (●) using the adsorbed serum is shown in A. Activity of an equal amount of unadsorbed serum against the same liposomes is shown in B. The data shown have been corrected for background activity by subtracting the values obtained for preimmune sera.

components as separate antigens, significant antibody activity remained in the adsorbed serum against DMPSC, but there was no reactivity with DMPC (Fig. 5). A slight apparent residual activity could be detected against CHOL, but the level of this activity was too low to determine if it represented significant binding activity (Fig. 5).

Discussion

In this paper we have described the production of antibodies to the bulk liposomal phospholipid, either DMPC or DMPSC, obtained after injection of the respective liposomes containing LA into rabbits. The chemical structure of DMPC differed from DMPSC by the replacement of the quaternary ammonium group of DMPC by a sulfonium group (Fig. 1). The antisera obtained reacted with both phospholipids and it was initially impossible to determine whether multiple populations of monospecific antibodies were induced or whether cross-reacting antibodies were present in the antisera. However, the possibility of multiple specificities was suggested by the observation that following the boosting immunization the antisera induced by immunizing with DMPSC showed much higher titers

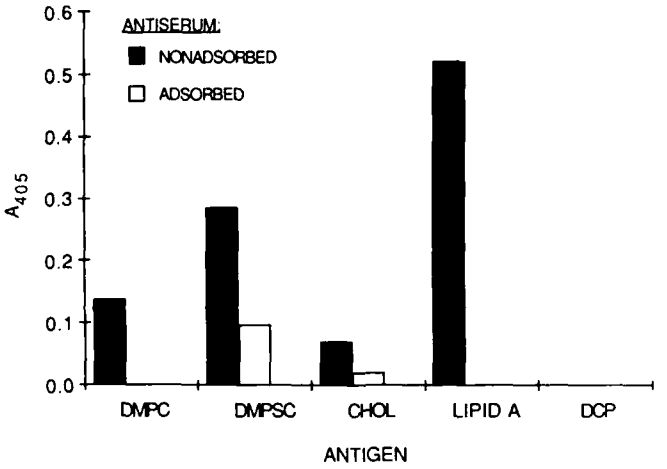


FIG. 5. Activities of adsorbed and nonadsorbed serum against each individual liposomal component, as determined by ELISA. Aliquots of serum from a rabbit immunized against DMPSC-CHOL-LA were adsorbed with liposomes lacking DMPSC, but containing DMPC, as described in Fig. 4. The activities of the resulting adsorbed antisera and of an equal amount of unadsorbed antiserum were determined by using a serum dilution of 1:25. Values reported have been corrected for background activity and nonspecific binding by subtracting absorbance values of preimmune sera.

against DMPSC compared with DMPC liposomes (Figs. 2B and 3B). It seemed likely that the apparent cross-reactivities exhibited by anti-DMPSC antiserum when tested with DMPC and DMPSC could have been due, at least partially, to different antibody populations. To test this hypothesis we adsorbed the antiserum with liposomes containing DMPC and tested for residual activity against DMPC or DMPSC liposomes (Fig. 4) or with the individual liposomal constituents as antigens in ELISAs (Fig. 5). It is clear that the antibody activity against DMPC, but not against DMPSC, was entirely removed by adsorption with DMPC (Figs. 4 and 5). The data indicate that we were dealing with populations of different antibodies, and a subpopulation could be identified that had the ability to distinguish DMPSC from DMPC.

The data presented in this report are therefore consistent with our previous studies which show that although liposomes function as complete antigens, the ability of antibodies to differentiate between liposomes having different, but similar, phospholipid compositions can be quite considerable. Specificities of antibodies raised in this fashion against individual phospholipids were previously observed with polyclonal antisera (Schuster *et al.* 1979; Alving 1986) or monoclonal antibodies (Banerji *et al.* 1982; Wassef *et al.* 1984). For example, a monoclonal antibody to PIP reacted 1000-fold more strongly with DMPC-CHOL-DCP-PIP liposomes than with DMPC-CHOL-DCP liposomes (Wassef *et al.* 1984). The present study demonstrates the utility of adsorbing antisera with liposomes to increase the specificity of antisera, by selectively removing populations of antibodies that cross-react with phospholipids that are closely similar to the immunizing antigen.

The ability to prepare specific antisera that recognize DMPSC, but not DMPC, complements previous research which has demonstrated that antibodies to phosphatidylcholine can be induced by utilizing liposomal LA as an

adjuvant (Schuster *et al.* 1979). Autoantibodies to phosphatidylcholine have also been induced by immunizing mice with bromelain-treated mouse erythrocytes (Cox and Hardy 1985), and monoclonal antibodies that recognize the trimethylammonium head group of phosphatidylcholine have been induced by this technique (Poncet *et al.* 1985). Antibodies to DPPC were also induced in guinea pigs by emulsifying DPPC with bovine serum albumin and complete Freund's adjuvant (Niedieck *et al.* 1987). The antisera to DPPC were inhibited by phosphocholine. A mouse monoclonal myeloma IgA anti-phosphocholine antibody (TEPC 15) induced by pristane reacted with phosphocholine and cross-reacted with lysolecithin and sphingomyelin, thus indicating specificity for the phosphocholine head group (Niedieck *et al.* 1987; Urbaneja *et al.* 1987). Differential reactivity of the TEPC 15 antibody occurred with monolayers of phosphatidylcholine and phosphatidylethanolamine (Urbaneja *et al.* 1987).

The present study supports the concept that considerable specificity can be exerted by antibodies directed against the head group of phosphatidylcholine. Replacement of the trimethylammonium group with dimethylsulfonium provided sufficient alteration of the head group to allow induction of antibodies that differentiated the two head groups. The antibodies that had such specificity were undoubtedly part of a larger population of antibodies that cross-reacted to varying degrees with both DMPC and DMPSC. These data, therefore, are compatible with the concept that the antigen binding sites of antibodies to phospholipids have capacities to recognize patterns of membrane surfaces. Binding of subpopulations of polyclonal antibodies can be influenced by subtle chemical changes of liposomal phospholipid polar head groups, and a high degree of specificity can be achieved by adsorption by antiserum with appropriate liposomes.

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